PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61M 11/00, A01N 37/44	A1	(11) International Publication Number: WO 97/18000
		(43) International Publication Date: 22 May 1997 (22.05.97)

(21) International Application Number: PCT/US96/17508

(22) International Filing Date: 31 October 1996 (31.10.96)

(30) Priority Data:
08/559,172 13 November 1995 (13.11.95) US

(71) Applicant: BRIGHAM AND WOMEN'S HOSPITAL [US/US]; 75 Francis Street, Boston, MA 02115 (US).

(72) Inventor: STAMLER, Jonathan; 3416 Juniper Place, Chapel Hill, NC 27514 (US).

(74) Agents: HERRON, Charles, J. et al.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: S-NITROSO-HEMOGLOBIN AND THERAPEUTIC USES THEREOF

(57) Abstract

Disclosed is a human hemoglobin in which at least one amino acid thiol moiety is S-nitrosylated, particularly one obtained by nitrosylating a thiol moiety on the single cysteine included in the sequence of one or both hemoglobin β subunits. Also disclosed are pharmaceutically acceptable compositions including S-nitrosylated hemoglobin and uses therefor.

AQ1

S-NITROSO-HEMOGLOBIN AND THERAPEUTIC USES THEREOF

This application is a continuation-in-part of U.S. application ser. no. 08/338,893, filed November 14, 1994 (copending) which is a divisional of U.S. application ser. no. 08/943,834, filed September 14, 1992 (now U.S. Patent No. 5,380,758) which is a continuation-in-part of U.S. application ser. no. 07/804,665, filed Dec. 11, 1992 (abandoned), which is a continuation-in-part of U.S. application Ser. No. 676,691, filed Mar. 29, 1991, (abandoned). This application is also a continuation-in-part of U.S. application ser. no. 08/198,854, filed February 7, 1994 (copending) which is a divisional of U.S. application ser. no. 07/943,835, filed September 14, 1992 (abandoned), which is a continuation-in-part of U.S. application ser. no. 07/791,668, filed November 14, 1991 (abandoned). This application is also a continuation-in-part of U.S. application ser. no. 08/409,720, filed March 24, 1995 (copending).

This invention was made with government support under RO1-HL40411, HL43344, and RO4870, awarded by the National Institutes of Health. The government has certain rights in the invention.

The need for an artificial blood substitute has become increasingly compelling because of periodic shortages of blood and the rising incidence of blood-borne illnesses such as AIDS and hepatitis. Notwithstanding the efforts of numerous investigators there are substantial obstacles that need to be overcome before any such product gains widespread use. Recent clinical trials have been terminated prematurely due to gastrointestinal, cardiovascular, and respiratory side effects: specifically, increases in blood pressure, painful abdominal symptoms, and arterial oxygen desaturation. Hemoglobin and other blood substitutes and oxygen delivery systems cause vascular constriction in the entire cardiovascular system, particularly in the lung and gastrointestinal tract by causing the contraction of vascular smooth muscle. Further, hemoglobins and other oxygen delivery systems that consume or scavenge nitric oxide thereby cause vasoconstriction which hinders oxygen delivery and thereby raises blood pressure. Overcoming these limitations would end a lengthy quest for an alternative to red blood cell transfusions and satisfy a strong medical need.

Several complications of blood substitute therapy, such as renal toxicity, short half-life in the circulation and excessively high oxygen affinity have been ameliorated by rational approaches to drug design, such as cross-linking of lower molecular weight dimers and subunits. The residual side effects of cell-free hemoglobin solutions appear to be the direct consequence of their high affinity binding of nitric oxide (NO). In this respect, NO produced locally in blood vessels, airways and the gastrointestinal tract regulates the pressure of the blood, airway tone, and the peristaltic contractions of the stomach, intestines and colon. Both enzyme inhibitors that selectively decrease NO production and hemoglobin which traps nitric oxide promote vasoconstriction, alterations in intestinal motility, and ventilation perfusion mismatching in animal models.

Cell-free blood substitute compositions sought to be used for treating hemorrhage and preserving organs and tissue for transplantation have predominantly been preparations of heme-containing proteins. Numerous investigators and foreign and domestic companies have sought to produce isolated human, bovine and other

hemoglobin and modified hemoglobins for use in cell-free blood substitute preparations. Native hemoglobin has been isolated from blood and used in such preparations.

Modified hemoglobins have been prepared and used for such cell-free preparations. For example, cross-linked polymerized hemoglobins (see U.S. Patent No. 5,194,590) and cross-linked polymerized pyridoxylated hemoglobin (U.S. Patent No. 5,194,590 and 4,826,811) have been prepared. Multimeric hemoglobin-like proteins based on pseudo-tetramer containing pseudo-dimer polypeptides with globin-like domains have been used in order to prolong hemoglobin half life (see WO 93/09143). Variants such as conjugates of hemoglobin with other drugs are also used (see-WO 93/08842). Di-alpha-globin-like polypeptide and di-beta-globin-like polypeptides connected into a single chain and incorporating heme have been used to prepare a human hemoglobin-like protein (see WO 90/13645). Hemoglobin mutants having lower O_2-affinity have been prepared by recombinant techniques and used as blood substitutes (see WO 88/09179).

Numerous other modifications of hemoglobins are disclosed in the patent literature for use as oxygen carrying compounds in blood substitutes. Hemoglobin and heme-containing protein based blood substitute compositions are disclosed, *inter alia*, in U.S. Patent Nos. 5,217,648; 5,194,590; 5,061,688; 4,826,811;5,281,579; 5,128,452; 5,248,766; 5,041,615; 4,861,867; 4,831,012; 5,296,465; 5,084,558; 5,295,944; 4,780,210; 4,925,574;5,264,555 and numerous others; and in PCT publication nos. WO 93/09143; WO 93/08842; WO 90/13645; WO 92/02239; WO 89/12456; WO 88/03408; WO 92/20369; WO 92/09630; WO 91/07190 and numerous others.

The endothelium secretes a vascular relaxing factor, known as endothelium-derived relaxing factor (EDRF), which has been identified as nitric oxide (NO), or a closely related derivative thereof. (Palmer *et al.*, *Nature*, *327*:524-526, 1987; Ignarro *et al.*, *Proc. Natl. Acad. Sci. USA*, *84*:9265-9269, 1987). Under physiologic conditions, however, NO is exceedingly unstable, reacting essentially instantaneously with oxygen, superoxide anion, and redox metals (Lancaster *et al.*, *Proc. Natl. Acad. Sci. USA*,

87:1223-1227, 1990; Ignarro *et al.*, *Circ. Res.*, *65*:1-21, 1989; and Gryglewski *et al.*, *Nature, 320*:454-456, 1986). This fact has lead to the supposition that, in order to exert its effect on vascular smooth muscle, NO must be stabilized *in vivo* in a form that preserves its biological activity.

S-nitrosothiols (RS-NO) are adducts that form readily under physiologic conditions from the reaction of NO with reduced low molecular weight thiols (Oae *et al.*, *Org. Prep. Proc. Int.*, *15(3)*:165-198, 1983). These compounds have half-lives that are significantly greater than that of NO and, like EDRF, possess vasorelaxant activity that is mediated, in part, through activation of guanylate cyclase (Kowaluk *et al.*, *J. Pharmacol. Exp. Ther.*, *256*:1256-1264, 1990; Loscalzo *et al.*, *J. Pharmacol. Exp. Ther. 249(3)*:726-729, 1989; and Ignarro *et al.*, *J. Pharmacol. Exp. Ther.*, *218(3)*:739-749, 1981).

As demonstrated by the inventors, S-nitrosylation of hemoglobin increases its oxygen-binding affinity. Hemoglobin is a globular protein, which binds reversibly to blood oxygen through passive diffusion from entry of air into the lungs. Hemoglobin-oxygen binding greatly increases the capacity of the blood to transport oxygen to bodily tissues; thus, the binding affinity between hemoglobin and oxygen is a critical factor in determining the level of oxygen transport to the tissues. The thiol group on the hemoglobin molecule regulates the affinity of hemoglobin for oxygen. The inventors have demonstrated that some S-nitrosothiols, such as certain S-nitroso-proteins and certain low molecular weight S-nitrosothiols do not react with the iron-binding site of hemoglobin, as does NO, but instead, bind to the thiol group. Thus, methemoglobin formation is prevented and hemoglobin-oxygen binding is unimpaired.

Furthermore, the inventors have also demonstrated that S-nitrosylation of hemoglobin not only prevents impairment of binding, but actually increases hemoglobin-oxygen binding. Therefore, another embodiment of the invention involves the administration of S-NO-hemoglobin or the *in vivo* nitrosylation of hemoglobin by

coadministration of a suitable S-nitrosothiol, to increase the oxygen-carrying capacity of the blood, and oxygen transport by hemoglobin to bodily tissues. As a result, these compounds may be useful in the treatment of disorders which are associated with insufficient oxygen transport, or in clinical situations in which increased oxygen transport is needed. Examples of such clinical situations include, but are not limited to, hypoxic disorders resulting from pneumothorax, airway obstruction, paralysis or weakness of the respiratory muscles, inhibition of respiratory centers by drug or other agents, or other instances of decreased pulmonary ventilation, including acute respiratory distress syndrome. Additional clinical indicates include impaired alveolar gas diffusion such as occurs in interstitial fibrosis, bronchiole constriction, pulmonary edema, pneumonia, hemorrhage, drowning, anemias, arteriovenous shunts, and carbon monoxide poisoning. In addition, nitroso-hemoglobin may also be used to modulate the delivery of carbon monoxide or nitric oxide (bound to hemoglobin) to bodily tissues. In addition, any thiol-containing heme proteins may be nitrosylated and used to enhance the oxygen-carrying capacity of the blood.

An additional aspect of the invention is the use of blood substitute compositions in accordance with the invention for maintaining and perfusing transplant organ or tissue materials such that they can be maintained for durations necessary to transport them or to culture expand them, particularly when they are largely comprised of progenitor cells. Such perfusion of organ and tissue maintenance compositions are in liquid form and constitute the compounds of the invention in physiologically acceptable carriers.

This invention relates to nitrosylation of blood substitutes, particularly including heme proteins such as hemoglobin as a therapeutic modality. The invention also relates to nitroso-protein compounds and their use as a means to selectively regulate specific protein functions, to selectively regulate cellular function, to endow the protein with new smooth muscle relaxant and platelet inhibitory properties and to provide targeted delivery of nitric oxide to specific bodily sites.

An additional aspect of the invention is blood substitute compositions comprising nitrosylated proteins and other constituents. These blood substitute compositions are preferably cell free. Principally useful in such compositions are various forms of nitrosylated hemoglobin and modified hemoglobins.

These nitrosylated blood substitutes have a vasorelaxant activity which is directly opposite to the vasoconstrictive properties of the non-nitrosylation forms. The administration of the nitrosylated forms effects vasodilation both by adding the administered nitric oxide to the recipient but also by not reducing through scavenging, the constitutive levels.

Additionally, the invention relates to nitrosylation of sites such as sulfhydryl (thiol), oxygen, carbon and nitrogen, present on proteins and amino acids, as a means to achieve the above physiological effects. The therapeutic effects may be achieved by the administration of nitrosylated proteins and amino acids as pharmaceutical compositions, or by nitrosylation of proteins and amino acids *in vivo* through the administration of a nitrosylating agent, such as in the form of a pharmaceutical composition.

Additionally, the invention provides a method for inhibiting the vasoconstrictive and nitric oxide depleting effects of hemoglobin and heme-containing based blood substitute compositions by the concurrent systemic administration of nitric oxide or a compound which donates, releases or transfers nitric oxide. Such administration can, for example, be by inhalation or intravenously, separately or in composition with the blood substitute.

In one aspect, the invention provides S-nitroso hemoglobin, preferably human hemoglobin, and particularly human hemoglobin which has been S-nitrosylated at the thiol moiety of a cysteine in one or both of the β-subunits of the hemoglobin. Thus, the invention particularly relates to a S-nitroso-hemoglobin obtained by the nitrosylation of

hemoglobin with an S-nitrosothiol. This compound may be used as a blood substitute or as a therapeutic agent to promote vasodilation and platelet inhibition, and to treat or prevent cardiovascular disorders. It does not suffer from the adverse effects of dangerously high blood pressure observed upon the administration of native hemoglobin.

Another aspect of the invention provides a method for enhancing the hypertension protective effect of administering S-nitroso-hemoglobin by co-administering a thiol, such as glutathione or N-acetyl cysteine. The NO groups of the SNO-hemoglobin will be transferred to the low molecular weight thiols which are more rapid and complete in their release and transfer of NO. Thus, the presence of these thiols will act as a catalyst for enhancing the hypotensive effect of the presence of NO.

In another aspect, the invention provides a composition comprising a human hemoglobin and a S-nitrosothiol. More particularly, the S-nitrosothiol is selected from the group consisting of S-nitrosocysteine, S-nitroso-N-acetylcysteine, S-nitrosohomocysteine, S-nitrosoglutathione and S-nitrosocysteinylglycine.

The invention also provides a method for systemic delivery of nitric oxide and for regulating oxygen delivery to bodily sites by administering pharmaceutical compositions containing S-nitroso-hemoglobin.

The invention also provides a method for increasing the capacity of hemoglobin to bind oxygen, comprising administering a therapeutically effective amount of an S-nitrosothiol compound to an animal in need thereof.

The invention also provides a method for increasing oxygen transport to bodily tissues, comprising administering a therapeutically effective amount of an S-nitrosothiol compound to an animal in need thereof.

The invention also provides a method for the treatment or prevention of disorders associated with insufficient oxygen supply to bodily tissues, comprising administering a therapeutically effective amount of an S-nitrosothiol to an animal in need thereof.

Further the invention provides a method of treating ischaemic syndromes, such as can applied as, for example, post-thrombolytic therapies. The S-nitrosylated-hemoglobins of the invention are effective to treat an individual in need of treatment for ischaemic injury. It is believed that they provide this effect by one or more mechanisms including increased oxygen delivery to the ischemic site by causing vasodilation and by scavenging uncharged nitric oxide and superoxide species from the circulation.

Further, the invention provided a method for the treatment of shock. Even though the administration of native hemoglobin has been accompanied by dangerous elevations in blood pressure, S-netrosylated-hemoglobin is effective in controllably elevating dangerously low blood pressures which are associated with shock. The degree to which a given dose of S-nitroso-hemoglobin will elevate a pathologically low blood pressure in a shock patient is proportional to the number of nitrosyl substitutions per hemoglobin molecule of the compound administered. One of ordinary skill in the art will be able to ascertain readily a dose and degree of polynitrosylation optimization for a standardized therapeutic preparation.

Figure 1: S-nitrosylation of hemoglobin, as determined by the Saville method.

Figure 2: UV spectrum of hemoglobin incubated with S-nitroso-N-acetylcysteine (SNOAC). Tracing 1 reflects the product of reacting 12.5μM oxyHb, with 12.5μM SNOAC, at time = 5 minutes. Tracing 2 reflects the product of reacting 12.5 μM oxyHb with 12.5 μM SNOAC, at time = 20 minutes. Tracing 3 reflects the product of reacting 12.5 μM oxyHb with 62.5 μM SNOAC, at time = 0. Tracing 4 reflects the product of reacting 12.5 μM oxyHb with 62.5 μM SNOAC, at time = 15 minutes.

Tracing 5 reflects the product of treating the S-nitroso-oxyhemoglobin, for example, from tracing with dithionitrite.

Figure 3: Reaction of nitric oxide at the iron-binding site of hemoglobin.

Figure 4: UV spectrum of S-NO-hemoglobin (Hb). Spectra show that oxygen binding to the heme site is largely unaffected at S-NO/Hb stoichiometries (0.05 and 0.37) that are lacking in smooth muscle contractile activity (see Figure 2). Higher ratios of S-NO/Hb (1.59) are associated with methemoglobin formation. Curves for oxy-Fe(II)-hemoglobin (dithionite-treated) and methemoglobin ($K_3Fe(CN)$-treated) are shown for comparison.

Figure 5: Reversal of oxyhemoglobin (Hb)-induced contraction of aortic rings by S-nitrosylation. Hemoglobin is shown to constrict vessels in a dose-dependent manner (•). S-NO-Hb, with a stoichiometry of 0.1 S-NO\Hb, entirely prevents the constrictor response (o). Increasing the S-NO-Hb\Hb stoichiometry to 1 and oxidizing the metal converts hemoglobin into a potent vasodilator (◆).

A significant advantage of S-nitrosothiols is that they deliver NO in its most biologically relevant, and non-toxic form. This is critical, because the pharmacological efficacy of NO depends upon the form in which it is delivered. As demonstrated by the inventors, S-nitrosothiols can deliver NO as charged species, nitrosonium (NO^+) or nitroxyl (NO^-), as opposed to the uncharged NO radical (NO•). This is important because the charged species behave in a very different manner from NO• with respect to chemical reactivity. In contrast to NO•, nitrosonium and nitroxyl do not react with O_2 or O_2 species to produce toxic oxides of nitrogen, and are also resistant to decomposition in the presence of redox metals. Consequently, administration of these NO equivalents does not result in the generation of toxic by-products, or elimination of the active NO moiety.

The term "nitrosylation" refers to the addition of NO to a thiol group (SH), oxygen, carbon or nitrogen by chemical means.

The term "regulated" means effective control of the activity of a protein or amino acid, in a selective manner so as to cause the protein or amino acid to exert a desired physiological effect.

The term "modified" means to effectively alter the activity of a protein or amino acid in a selective manner, so as to cause the protein or amino acid to exert a desired physiological effect.

The term "enhanced" means to alter effectively the activity of a protein or amino acid in a selective manner, so as to cause an increase or improvement in the activity of the protein or amino acid, or endow the protein or amino acid with additional capabilities.

The terms "mutant", "variant" or "fragment" refer to any structurally modified protein or polypeptide that retains the same physiological activity of interest, as the parent protein or polypeptide, whether to a greater or lesser extent. Other properties or advantages may be present in such mutant, variant or fragment compound, such as increased half-life or resistance to natural inhibitors of the parent protein. Mutation can refer, for example, to changes in one or more amino acids in the primary sequence. Variants can refer for example, to posttranslational modifications, such as glycosylation, conformational contraint, or the addition of lipid moieties. Fragments can refer to polypeptides that retain some, all or more of the desired physiological property of the parent protein while not having one or more amino acids or sequences thereof present in the parent protein.

The term "substantially homologous," refers to amino acid sequences of hemoglobins, modified hemoglobins and other heme-containing proteins, means that a

particular subject sequence, for example, a mutant sequence, varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between reference and subject sequences. For purposes of the present invention, sequences having greater than 90 percent homology, equivalent biological activity, and equivalent expression characteristics are considered substantially homologous. For purposes of determining homology, truncation of the mature sequence should be disregarded. Sequences having lesser degrees of homology, comparable bioactivity, and equivalent expression characteristics are considered equivalents. Other embodiments include fusion protein products that exhibit similar oxygen carrying activities. Further embodiments include such proteins that are chemically synthesized as well as any proteins or fragments thereof that are substantially homologous.

The term "activity" refers to any action exerted by the protein or amino acid which results in a physiological effect.

The pharmaceutical compositions utilized in this invention can be administered by intranasal, oral, enteral, sublingual, rectal, intramuscular, intravenous, or subcutaneous means.

The compounds of this invention can be employed in combination with conventional excipients; i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral or enteral application which do not deleteriously react with the active compounds. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, pethroethral fatty acid esters, hydroxymethylcellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents. emulsifiers, salts for influencing osmotic pressure, buffers,

colorings, flavoring and/or aromatic substances and the like which do not deleteriously react with the active compounds.

For parenteral application, particularly suitable vehicles consist of solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets dragees or capsules having talc and/or a carbonhydrate carrier binder or the like, the carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release composition can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

It will be appreciated that the actually referred amounts of active compounds used will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application and the particular site of administration. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art, using conventional dosage determination tests conducted with regard to the foregoing guidelines.

According to the present invention, a "therapeutically effective amount" of a pharmaceutical composition is an amount which is sufficient to achieve the desired pharmacological effect. Generally, the dosage required to provide an effective amount of the composition, and which can be adjusted by one of ordinary skill in the art, will vary, depending upon the age, health, physical condition, sex, weight and extent of disease, of the recipient. Additionally, the dosage may be determined by the frequency of treatment and the nature and scope of the desired effect.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to it fullest extent. The following examples are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire text of all publications cited above and below are hereby incorporated by reference.

Example 1

S-nitrosothiols Increase Hemoglobin-oxygen Binding

Experiments were conducted to evaluate the reaction between S-nitrosothiols and hemoglobin. S-nitrosylation of hemoglobin was accomplished by reacting 12.5 μM hemoglobin with 12.5 μM S-nitroso-N-acetylcysteine (SNOAC) for 5 and 20 minute intervals, or with 62.5 μM SNOAC with absorbance readings immediately (time = 0) and after 15 minutes. A sample of S-nitroso-oxyhemoglobin was also treated with dithionitrite. S-nitrosylation was verified, using standard methods for detection of S-nitrosothiols (Saville, *Analyst*, *83*:670-672 (1958)). The Saville method, which assays free NO_x in solution, involves a diazotization reaction with sulfanilamide and subsequent coupling with the chromophore N-(naphthyl)ethylenediamine. The specificity for S-nitrosothiols derives from assay determinations performed in the presence and absence of $HgCl_2$, the latter reagent catalyzing the hydrolysis of the S-NO bond. Confirmatory evidence for S-nitrosothiol bond formation was obtained by spectrophotometry, demonstrated by the absorption maximum of 540 nm, as shown in Fig. 1. This was demonstrated using NO^+ equivalents in the form of SNOAC. The low molecular weight S-nitrosothiols are then removed by desalting across G-25 columns.

Figure 2 shows spectra of hemoglobin which has been incubated with S-nitroso-N-acetyl cysteine (SNOAC). Tracing 1 reflects the product of reacting 12.5 μM oxyHb with 12.5 μM SNOAC, at time = 5 minutes. Tracing 2 reflects the product of reacting 12.5 μM oxyHb with 12.5 μM SNOAC, at time = 20 minutes. Tracing 3 reflects the

product of reacting 12.5 μM oxyHb with 62.5 μM SNOAC, at time = 0. Tracing 4 reflects the product of reacting 12.5 μM oxyHb with 62.5 μM SNOAC, at time = 15 minutes. Tracing 5 reflects the product of treating the S-nitroso-oxyhemoglobin, for example, from tracing 1, with dithionitrite.

In Figure 2, the peaks at 350 nm are indicative of the presence of S-nitrosothiols. The peaks at 400-450 nm are known as Soret peaks and they are indicative of the oxidative state of hemoglobin. The small doublets at about 550 nm show that O_2 is binding at the hemoglobin metal center and that, therefore, its oxygen binding capacity is unchanged. Tracing 5 shows a plateau, rather than a doublet, at this wavelength. This is because dithionitrite deoxygenates oxyhemoglobin and breaks down S-nitrosothiols to NO. NO disrupts O_2 binding, whereas S-nitrosothiols do not. This plateau indicates the shift from oxyhemoglobin to nitrosylhemoglobin.

For the purposes of comparison, equimolar concentrations of hemoglobin and $NaNO_2$ were reacted in 0.5N HCl, to form nitrosyl-hemoglobin, and the UV spectrum was obtained. As shown in Fig 3, NO reacted instantaneously with the redox metal site on hemoglobin.

S-nitrosylation of hemoglobin does not result in the formation of methemoglobin and consequent impairment in hemoglobin-oxygen binding. Furthermore, an additional experiment demonstrated that S-nitrosylation of hemoglobin causes a leftward shift in the hemoglobin-oxygen association curve, indicating an increase in oxygen binding. Thus, the reaction between S-nitrosothiols and hemoglobin not only eliminates the inhibition of oxygen binding which occurs from the reaction with NO•, but actually increases binding and oxygenation of the blood.

In summary, S-nitrosothiols are important intermediates in the metabolism of organic nitrates and endogenously-derived NO. Furthermore, these compounds provide

greater stability, a longer half life than NO, and retain its cyclic GMP-dependent bioactivity in blood vessels.

In addition, S-NO-hemoglobin may also be used to modulate the delivery of carbon monoxide or nitric oxide (bound to hemoglobin) to bodily tissues.

Example 2

S-Nitrosylation of Hemoglobin β93 Sulfhydryl

Our aim in these experiments was to design conditions which support selective S-nitrosylation of hemoglobin's β93 sulfhydryl without impairment of the oxygen delivery functionality. Our results indicate the feasibility of this reaction under physiological conditions (Figure 4). This is achieved by treating hemoglobin with agents which preferentially donate NO^+ (which targets SH groups), rather than NO• (which reacts preferentially with metals).

Hemoglobin is S-nitrosated by incubation with an alkylnitrite (*e.g.*, amyl nitrite or tert-butyl nitrite) or a thionitrite (e.g., S-nitroso-glutathione, S-nitroso-penicillamine, S-nitroso-cysteine and S-nitroso-N-acetylcysteine) for periods of one minute to one hour. The pH is optimized within the range of 6.5 to 7.5 to achieve stoichiometric S-nitrosylation. To avoid the potential formation of methemoglobin the reactions are preferably performed anaerobically. It may also be desired to perform the above procedure in saturating carbon monoxide solutions and under increased atmospheric pressures (*i.e.*, 5 atmospheres) to prevent interaction of NO with the heme site.

Figure 4 shows the UV spectrum of S-NO-hemoglobin (Hb). The spectra show that oxygen binding to the heme site is largely unaffected at S-NO/Hb stoichiometries (0.05 and 0.37) that are lacking in smooth muscle contractile activity. Higher ratios of S-NO/Hb (1.59) are associated with methemoglobin formation. Curves for oxy-Fe(II)-hemoglobin (dithionite-treated) and methemoglobin ($K_3Fe(CN)$-treated) are shown for comparison.

Synthesis of S-NO-hemoglobin is monitored by three complementary methods, two of which were developed in this laboratory: photolysis-chemiluminescence and capillary electrophoresis methodologies. The ligand binding to

heme is determined spectrophotometrically, using published extinction coefficients. The structure of S-nitrosylated hemoglobin is then further characterized using SDS-polyacrylamide gel electrophoresis and isoelectric focusing immunoelectrophoresis.

The photolysis-chemiluminescence technique (U.S. Patent No. 5,459,076) can be used to measure both protein and low molecular weight RS-NO. The sample protein is introduced directly or as a chromatographic effluent from an attached FPLC/HPLC (for separation and identification of protein and amino acid RS-NO, respectively) into a photolysis cell where it is irradiated with a 200 watt mercury vapor lamp, designed to result in complete photolysis of the S-N bond. NO is then carried in a stream of helium towards the reaction chamber which yields the chemiluminescence signal. To further ensure that signal originate from RS-NO, measurements are compared before and after treatment with $HgCl_2$, which selectively displaces NO from thiol groups. We have confirmed that metal-nitrosyl complexes are not affected by this treatment.

In the Saville assay method, NO^+ displaced from RSNO with Hg^{2+} ion is assayed by diazotization of sulfanilamide and subsequent coupling to the chromophore N-[1 naphthyl] ethylenediamine.

The capillary electrophoresis method (U.S. Patent No. 5,346,599) offers the added advantage of being able to separate hemoglobin variants as well as various RS-NO from their respective thiols and disulfides. We have recently reported on simultaneous detection of RS-NO derivatives of cysteine, glutathione and homocysteine, together with their respective thiols and disulfides, using this technique.

Example 3

Bioassays of S-NO-Hemoglobin

The adverse clinical consequences of cell-free hemoglobin result from its contractile effects on blood vessels, airways, and intestinal smooth muscle. *In vitro* bioassays are, therefore, used to assess the smooth muscle relaxant properties of S-NO hemoglobin in these tissues. Such organ chamber experiments are routinely performed to assess the effects of NO donors on blood vessels, airways and intestinal tissues.

Methods for preparation of blood vessels, airways and intestinal smooth muscle are performed as previously described. Briefly, descending thoracic aorta, trachea, or intestinal tissues are isolated from anesthetized New Zealand white female rabbits. The tissues are cleaned, cut into small rings and mounted on stirrups connected to force transducers by which changes in isometric tension are recorded. Sustained contractions are elicited with agonists (i.e., histamine or acetylcholine for airway smooth muscle or phenylephrine in the case of blood vessels), or by electrical field stimulation.

Preliminary experiments showing that S-NO-hemoglobin is a potent relaxant of phenylephrine contracted aortic rings is shown in Figure 5.

Figure 5 shows reversal of hemoglobin (Hb)-induced contraction of aortic rings by S-nitrosylation. Hemoglobin is shown to constrict vessels in a dose-dependent manner (•). S-NO-Hb, with a stoichiometry of 0.1 S-NO\Hb, entirely prevents the constrictor response (o). Increasing the S-NO-Hb\Hb stoichiometry to 1 converts hemoglobin into a potent vasodilator (◆). Moreover, these studies show that S-nitrosylation of hemoglobin abrogates the contraction induced by the native protein.

Example 4

Oxygen Affinity of S-NO-Hemoglobin

The successful development of hemoglobin based blood substitutes involves and understanding of oxygen binding capability. In addition to classical influences on oxygen affinity (*i.e.*, 2, 3, DPG, pH and CO_2), alkylation of the β93 SH group(s) of hemoglobin shifts the O_2 hemoglobin association curve leftward. For this, we evaluate the effects of S-nitrosylation on native and cross-linked hemoglobins, as well as other hemoglobin variants. A comparative analysis of the effects of S-nitrosylation, alkylation (i.e., with N-ethylmaleimide) or oxidation (*i.e.*, with reactive disulfides such as dithionitrobenzoic acid) advance our understanding of the molecular control mechanisms of oxygen delivery.

Measurement of highly precise oxygen binding curves is performed using standard tonometry, an Imai cell and/or a thin-layer Gill cell modified with an oxygen electrode. For studies of oxygen binding by intact red blood cells, with or without added cell-free hemoglobins, a thin layer dual-beam method is used. For these measurements we use a modified Hemoscan (American Instruments Co.) operated in a discontinuous mode to avoid dynamic error.

Example 5

In Vivo Vasorelaxant Studies

Rabbits are routinely used in the laboratory for assessment of hemodynamic responses to pharmacological agents. In addition, we use a superfused guinea pig lung model to assess the effects of NO donors on airway resistance. It is well known that hemoglobin infusions increase blood pressure in rabbits and would be predicted to modestly increase airway tone.

Our objectives were, therefore, to demonstrate vasorelaxant and bronchodilator properties of S-NO-hemoglobin, determine the biological half life of the molecule and obtain insight into the degree of S-nitrosation required to overcome its contractile effects. In particular, the data obtained in ring studies *in vitro* indicates that very small degrees of S-nitrosation are required to ameliorate vasoconstriction. Specifically, one NO per 40 heme groups is sufficient to prevent aortic smooth muscle contraction.

Hemodynamic Measurements

New Zealand white rabbits were anesthetized with ketamine (50 mg/kg i.m.) and sodium barbital (5-10 mg/kg i.v.) after which the femoral artery was cannulated to allow continuous measurement of blood pressure. Mean arterial pressure was then measured in response to intravenous bolus injections of S-NO hemoglobin (1 nmol/kg/min to 1 μm/kg/min) as well as continuous infusions. Blood pressure did not rise whereas native hemoglobin caused a hypertensive effect.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A human hemoglobin, including mutants, variants and fragments thereof, in which at least one amino acid thiol moiety is S-nitrosylated.

2. The human hemoglobin of claim 1 wherein at least one amino acid thiol moiety is on the single cysteine included in the sequence of a hemoglobin β subunit.

3. The human hemoglobin of claim 1 wherein at least one amino acid thiol moiety is on the single cysteine included in the sequence of each of the two β subunits of human hemoglobin.

4. A composition comprising human hemoglobin and an S-nitrosothiol in a pharmaceutically acceptable carrier.

5. The composition of claim 4 wherein the S-nitrosothiol is selected from the group consisting of S-nitrosocysteine, S-nitroso-N-acetylcysteine, S-nitrosohomocysteine S-nitrosocysteinylglycine, and S-nitrosoglutathione.

6. An S-nitrosylated human hemoglobin obtained by nitrosylating at least one amino acid thiol moiety of a human hemoglobin.

7. The S-nitrosylated human hemoglobin of claim 6 obtained by nitrosylating a thiol moiety on the single cysteine included in the sequence of a hemoglobin β subunit.

8. The S-nitrosylated human hemoglobin of claim 6 obtained by nitrosylating a thiol moiety on the single cysteine included in the sequence of each of the two β subunits of human hemoglobin.

9. The S-nitrosylated human hemoglobin of claim 6 obtained by nitrosylating at least one amino acid thiol moiety of a human hemoglobin with an S-nitrosothiol.

10. The S-nitrosylated human hemoglobin of claim 6 obtained by nitrosylating at least one amino acid thiol moiety of a human hemoglobin with an S-nitrosothiol selected from the group consisting of S-nitrosocysteine, S-nitroso-N-acetylcysteine, S-nitrosohomocysteine, S-nitrosoglutathione and S-nitroso-cysteinylglycine.

11. A method for treating ischaemic injury or shock in an individual in need thereof by administering to said individual a therapeutically effective amount of an S-nitroso-hemoglobin.

FIG. 1

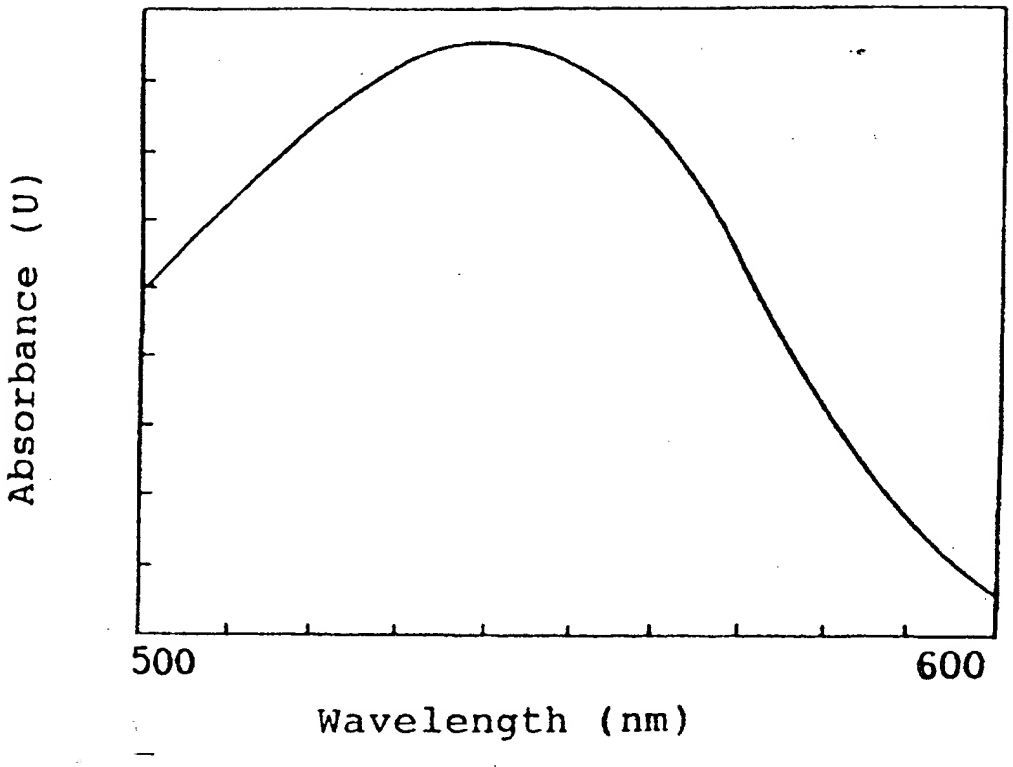


FIG. 2

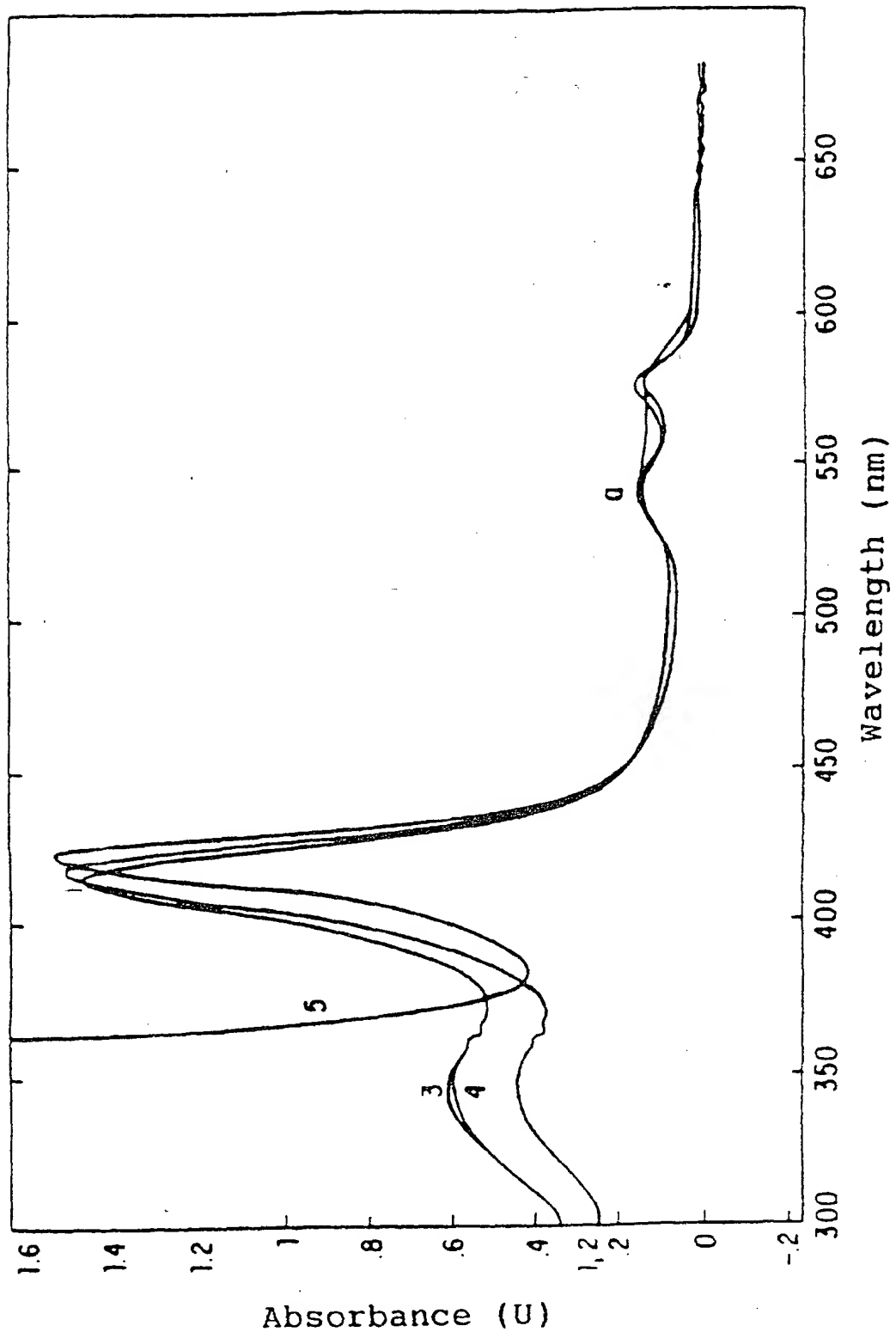

Absorbance (U)
1.6
1.4
1.2
1
.8
.6
.4
1,2
.2
0
-.2
Wavelength (nm)
300
350
400
450
500
550
600
650
a
3
4
5

FIG. 3

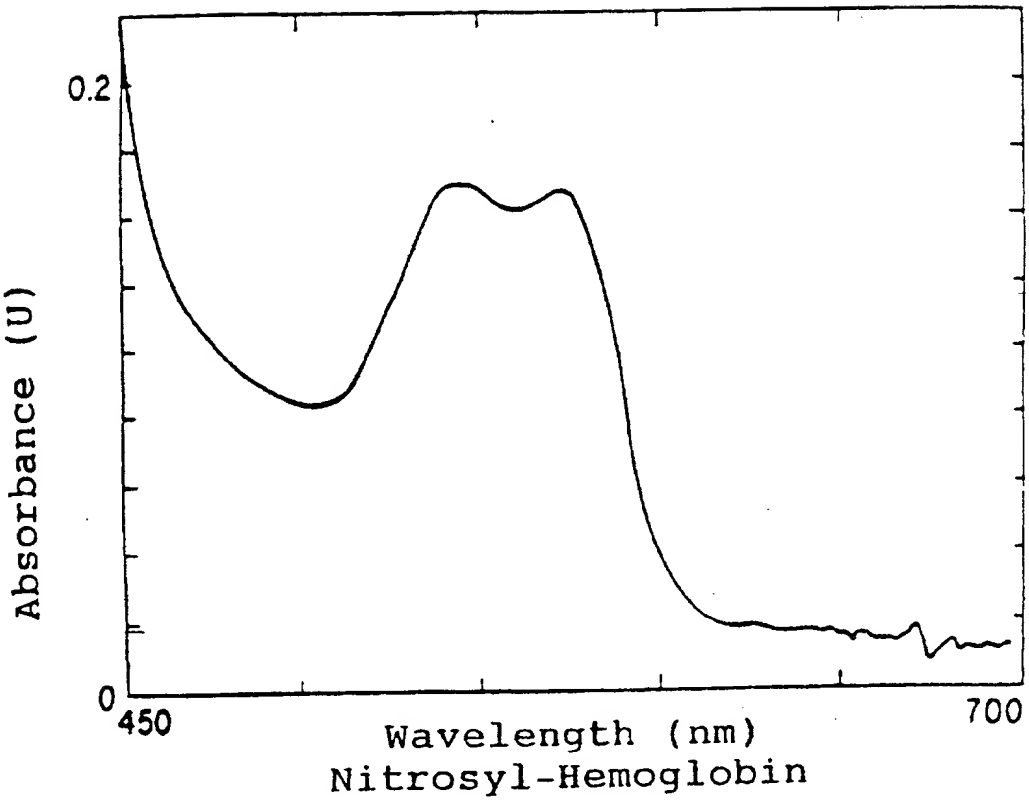

0.2
0
Absorbance (U)
450
700
Wavelength (nm)
Nitrosyl-Hemoglobin

FIG. 4

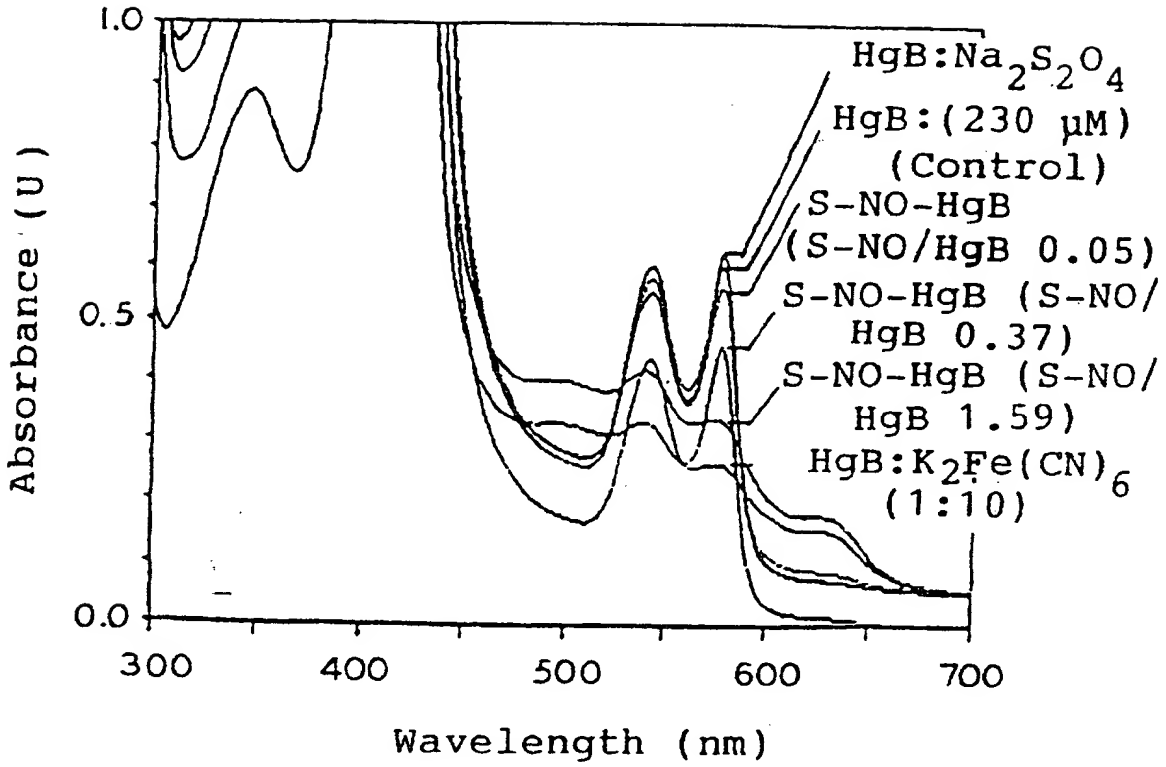
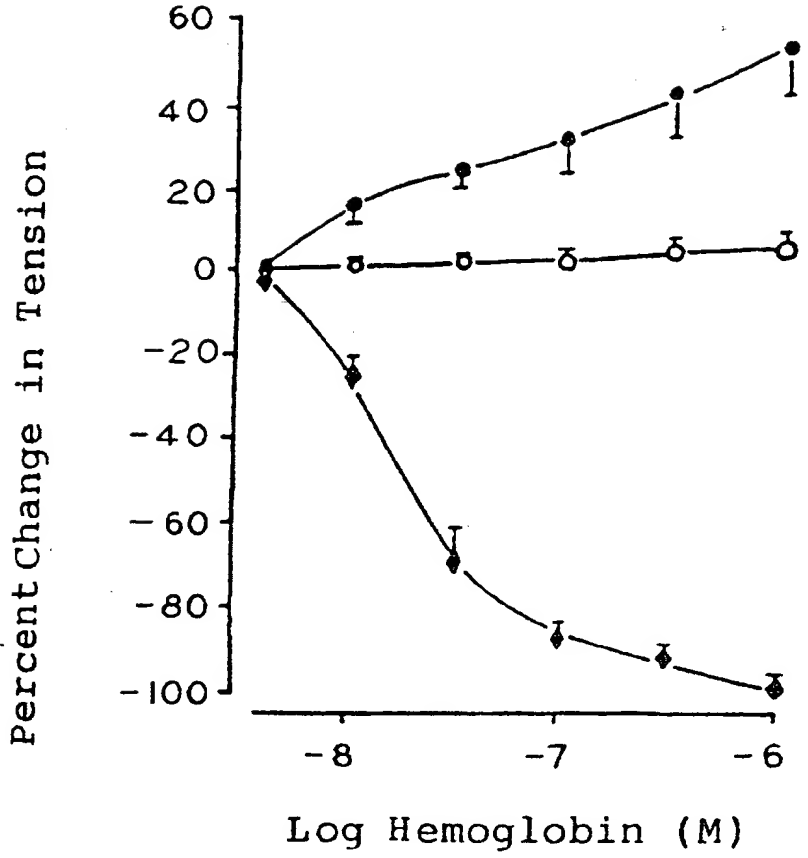

1.0
0.5
0.0
Absorbance (U)
300
400
500
600
700
Wavelength (nm)
HgB:Na2S2O4
HgB:(230 µM)
(Control)
S-NO-HgB
(S-NO/HgB 0.05)
S-NO-HgB (S-NO/
HgB 0.37)
S-NO-HgB (S-NO/
HgB 1.59)
HgB:K2Fe(CN)6
(1:10)

FIG. 5


60
40
20
0
-20
-40
-60
-80
-100
Percent Change in Tension
-8
-7
-6
Log Hemoglobin (M)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/17508

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61M 11/00; A01N 37/44
US CL :514/562; 128/200.14, 200.15, 200.23
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/562; 128/200.14, 200.15, 200.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US, A, 5,485,827 (ZAPOL ET AL.) 23 January 1996, see entire disclosure.	1-11
X	Archives of Biochemistry & Biophysics, Vol. 303, No. 2, issued June 1993, Alayash et al., "Nitric Oxide Binding to Human Ferrihemoglobins Cross-Linked Between Either α or β Subunits", pages 332-338, see entire disclosure.	1-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
23 JANUARY 1997	11 FEB 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DR. HERBERT J. LILLING Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*